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Investigation of the flavonoidal constituents and insecticidal activity of *Teucrium zanonii*

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ABSTRACT

Teucrium zanonii (Family labiateae) is an endemic Libyan plant growing in many regions around Benghazy city. Investigation of the flavonoidal constituents of plant led to isolation of Cirsiliol, Luteolin, Chrysoeriol and Xanthomicrol from ethyl acetate fraction while Apigenin 6,8-di-O-glucoside and Luteolin-7-O-rutinoside were isolated from the butanol fraction. All compounds were identified by chromatographic and spectroscopic methods (UV, MS, ^{1}H and ^{13}C NMR spectroscopy). The study of insecticidal activity of different extracts of the plant against the adult of Phloeotribus oleae showed the highest effect (86.67% mortality) was observed with the aqueous extract, while the unsaponifiable fraction was the least in this concern which give only 43.33% mortality,. Also, mortalities of 83.33%, 80.00%, 70.00% and 66.67% were obtained by using of alcoholic, butanol, ethyl acetate and chloroform extracts, respectively. Also the filed experiments proved that aqueous, alcoholic and butanol extracts significantly lowered the percentage of infestation to 70.82%, 65.86% and 66.56%, respectively after one week.

Keywords: *Labiateae, Teucrium zanonii, flavonoids, Phloeotribus oleae,* insecticidal activity .

INTRODUCTION

The family *Lamiaceae* (Labiatae) is commonly called the mint or aromatic family due to high content of essential oils with aromatic odor. It is one of the flowering plant groups (1) . In the most recent classification the family comprises about 252 genera and 6700 species. In Libya the family is represented by 22 genera and 65 species (2).Lamiaceae species are important in the field of pharmacology, cosmology perfumes and food industry. Many species have great potential in the preparation of drugs in modern medicine (3–4).

The genus *Teucrium* belonging to *Lamiaceae* family is represented by about 300 species in the world. It represented by 13 species in the flora of Libya, five of them being endemic, *viz.; T. appollinis, T. barbeyanum, T. davaeanum, T. linivaccarii, and T. zanonii* in which *T. zanonii* is the most common one(2). Chemical investigation of the plants this genus led to isolation of sesquiterpenes, triterpenes, sterols, flavonoids, iridoids, phenolic acids and some alkaloids (5–8). Many *Teucrium* species have been used for more than 2000 years as medicinal plants. They exhibit some interesting biological activities like diuretic, diaphoretic, antiseptic, antipyretic, antispasmodic, hypoglycemic, antifeedant, besides some of *Teucrium* extracts are used in folk medicine to treat various aliments such as stomach and intestinal troubles, cold and as stimulant vermifuge, tonic, rheumatism, hemorroids and renal inflammatory (9–12).

Olive trees are liable to infestation by several destructive borers. The olive bark beetle, *phloeotribus oleae* Fab. (Order: *Coleoptera*, Family: *Scolytidae*) is considered one of these

borers, since it cause serious damage to olive trees which may lead them to death within few years. These losses start when the female beetle deposits her eggs beneath the bark of the tree. The hatching grubs complete their development in the cambium region, and then the beetles emerge through small round holes. Various conventional chemical insecticides are available which offer some protection against this pest, but they have created many problems such as resistance, secondary pests outbreaks, environmental contamination …etc. (13).

A promising alternative in this regard is the application of plant extracts which can be both effective and inexpensive. In the framework of our chemical and biological investigations of Libyan medicinal plants, this work aimed to investigate the flavonoidal constituents and insecticidal activity of different extracts of *T. zanonii*.

MATERIAL AND METHOD

Plant Material

The plant was collected from Abo-fakhra region about (25 Km) from Benghazi city in April 2005 during the flowering stage. The plant was kindly identified by Dr. Mohamed Alsharif at Botany department, Faculty of science, Gariuones University. A voucher specimen has been deposited at the Herbarium of Biology department, Faculty of science, Altahady University, Sirt, Libya.The aerial parts of the plant (leaves, flowers and branches) were air dried and ground altogether till it become as a fine powder.

Preparation the plant extracts for biological evaluation

About 100g of dry powered plant were extracted with petroleum (40–60°C) in a Soxhlet apparatus. The petroleum ether was fractionated to fatty alcohols, fatty acids and unsaponifide materials. The defatted plant material was extracted with aqueous methanol (70%). The alcoholic extract was partitioned with chloroform, ethyl acetate and butanol, respectively. Another 100g of the plant material were macerated in distilled water for 24 hours. The tested extracts were petroleum ether, fatty alcohols, fatty acids, unsaponifide fraction, alcoholic, aqueous, chloroform, ethyl acetate and butanol extracts.

Stock culture

Cutting of olive branches severely infested with the olive bark beetle, *Phloeotribus oleae* Fab. Were collected from Burg-El-Arab region, Matrouh Governorate, Egypt.

A box of 60 × 60 × 100 cm, walls and floor was constructed of wooden frames covered with wire gauze and lined with cloth streamers while the ceiling and the door were made of glass. The collected cutting of olive branches were left under laboratory condition (25±2°C and 65± 5% R.H.), until emergence of the beetles at about the beginning of March. Newly emerged addults were collected and classed according to sex , Cutting of fresh olive branches 10 cm long and 2.5 cm thick, each were used as an oviposition site. They were left for 1–3 days to be suitable to the entry of beetles.

Control experiments
Laboratory tests

They were conducted using nine plant extracts, the slide dip technique (14) was used .A small piece of a double faced adhesive tap was adhered on a glass slide and ten adult beetles of *P. oleae* of the same age were transferred by means of a soft brush and placed with their backs on the surface of the tape. The slide with the adults on it was dipped for 5 seconds in each extract after which the slides were left to dry under room temperature. The beetles of the untreated control were dipped in water for comparison. Three replicates were used for each extract and untreated control, the dead and alive beetles were recorded after 24 hours.

Field experiment

An orchard of olive trees naturally infested with the fore mentioned insect was chosen at Burg El-Arab region, Matrouh Governorate. The trees were 6–8 years old and about 1.5–2.0 m in a height. For the individual extract, a randomized block design was used where nine treatments were arranged in three replicates, 4 trees each (12 trees/ treatment). Rows of olive trees were left, as borders among the treatments, to avoid any spray drift. All spray applications were made once on late March, using knapsack sprayer 20 L capacity, to cover stems, branches and twigs of all trees. Five of green branches, were randomly selected from each tree, and cut off. Then they were kept in plastic bags and transferred to the laboratory where they were examined. The number of living adults per branch either before or after application was recorded in each treatment (including control plats) or used as an index for the population density (infestation) of the borer. Pretreatment counts were taken immediately before spraying application, whereas post treatment counts were taken 1, 2 and 3 weeks after application.

Evaluation of all treatments was based on the reduction of the population density of olive individuals per replicate according to Henderson and Tilton equation (1955) (15). Data were statistically analyzed using Duncan's Multiple range test (1955) (16).

Extraction and fractionation of the flavonoidal constituents

About 1kg of the air dried powdered plant of *T. zanonii* was defatted with petroleum ether (b.r.40–60°C) (5 L). The defatted powder was macerated with aqueous methanol (70%) till exhaustion. The alcoholic extract was evaporated in *vacuo* at about 50°C (73.7 g), dissolved in hot distilled water (300 ml), left overnight in refrigerator and then filtered. The aqueous filtrate was extracted with successive portion of ethyl acetate (5×500 ml) followed by butanol (5×500 ml). The solvents were dried; separately; over anhydrous sodium sulphate and evaporated in *vacuo* at 50 °C. The ethyl acetate and butanol free residues amounted to 3.5g and 6.5 g respectively.

Fractionation of the ethyl acetate extract of *T. zanonii*

About 3 g of the ethyl acetate extract were dissolved in 5 ml of methanol: water (80: 20) and applied on the top of Sephadex LH-20 column preswollen in aq. Methanol (80:20) for one night. Elution was affected with methanol: water with decreasing the polarity. Fractions 25 ml each were collected and the course of chromatographic fractionation was followed using PC in 15% acetic acid as a developing solvent.

Purification of flavonoidal compounds

The fractions 27–35 containing compound-1 were collected and rechromatographed over small column of Sephadex LH-20 eluted with methanol: water (90: 10), and collecting small fractions (10 ml). The fractions containing compound-1 in pure form were collected (10mg). Compound-2 was obtained from fractions 36-50 in pure form.

The fractions 21–26 were collected and it was found to contain two main flavonoidal compounds. It was applied into a preparative thick layer chromatography (PTLC) using chloroform-methanol (80:20) as a developing solvent system. Two main zones (R_f 0.52 and 0.68) were localized under UV light, scrapped off and eluted with methanol (90%). The methanol was evaporated from each zone to afford two compounds (3 and 4) in pure form but in small quantities (2.7 mg and 2.5 mg respectively).

Investigation of butanol extract

About 6.5 g of butanol fraction were subjected to preparative paper chromatography (PPC) and developing with 25% acetic acid. Two main zones (I, II) were localized under UV light, cut into small pieces and eluted, separately, with methanol 70%. The methanol was evaporated under reduced pressure at 45°C to afford two impure compounds (5 and 6).

The residue obtained from zone-I (compound-5) was further purified by PPC, developed with BAW (4:1:5). Finally it was purified by passing through small Sephadex LH-20 column, eluted with 70% methanol to give compound-5 in pure form (2DPC, different solvent system).

The residue obtained from elution of zone-II was further purified using PPC developed with 20% acetic acid. The main zone was eluted as before and the obtained residue was further purified in another solvent system (B: A: W. 3:1:1). The pure compound-6 was eluted and passed over small column of Sephadex LH-20 column eluted with methanol (70 %).

Acid hydrolysis

About 5 mg of bothcompound-5 and 6 were dissolved in 25 ml of 2N HCl : MeOH (1:1) separately and refluxed on boiling water bath for 2 hours. After complete hydrolysis, the solvent was evaporated and diluted with distilled water. The aglycone was extracted with ethyl acetate (3 × 50 ml). The ethyl acetate extract was washed with distilled water till free from acidity. The aglycone was obtained after evaporation of the solvent. The aglycone was further purified by passing over a small Sephadex LH-20 column, eluted with methanol. The aqueous layer after removal of the aglycone was rendered neutral with Barium carbonate, filtered, evaporated and dissolved in isopropanol. The mixture of sugars was investigated by PC using Phenol saturated with water as developing solvent against some authentic sugars. The chromatograms was dried and sprayed with aniline phathalate reagent and heated in an oven at 110°C for 5 min (17).

RESULTS

Compound-1:uv, λ_{max}(MeOH) 254 (sh), 274, 344, (NaOMe) 270, 396, ($AlCl_3$) 275,300,340,420, ($AlCl_3$/HCl) 263,283,366, (NaOAc) 268,402 (NaOAc/H_3BO_3) 261,367. MS: at m/z=330(M^+), 329(M^+ −1)315(M^+− Me), 287(M^+ −[CO+Me]),196(A^+_1) and 134(B^+_1). ^{1}H-NMR (CD_3OD) showed signals 7.45 (1H, d, H-6′), 7.41 (1H, d, H-2′), 6.91 (1H, s, H-8), 6.80 (1H, d, H-5′), 6.61 (1H, s, H-3), 3.98 (3H, s, C-7-OCH_3), 3.83 (3H, s, C-6-OCH_3).

Compound-2 : uv,λ_{max}(MeOH)253,265,348,(NaOMe) 265,330(sh),400,($AlCl_3$)272,300,331, 422,($AlCl_3$/HCl) 262,275,293,356,383(NaOAc)267,329,396,(NaOAc/H_3BO_3)259,300,370. MS: at m/z=286(M^+), 315(M^+ − Me), 258(M^+ −CO),153(A^+_1+1) and 134(B^+_1)

Compound-3: uv λ_{max} (MeOH) 255, 267, 291, 340, (NaOMe) 271, 309, 388, ($AlCl_3$) 257, 275, 294, 345, 386, ($AlCl_3$/HCl) 256, 276, 294, 346, 380

(NaOAc)273,312,381,(NaOAc/H_3BO_3)267, 343.MS:at m/z=300(M^+), 315(M^+ − Me), 269(M^+ − OMe]),241(M^+ − [CO+OMe]), and 148(B^+_1)

Compound-4: uv λ_{max} (MeOH) 274, 333, (NaOMe) 272, 385, ($AlCl_3$)263, 288, 298, 360,($AlCl_3$/HCl) 263, 288, 298, 354, (NaOAc) 271, 389

(NaOAc/H_3BO_3) 273, 336. MS:at m/z=344(M^+), 329(M^+− Me), 314(M^+ -HCHO),298(M^+ − OCH_3+CH_3) and 118(B^+_1).

Compound-5: uv λ_{max} (MeOH) 272, 330, (NaOMe) 281, 332, 399 ($AlCl_3$) 279, 304, 349, 383, ($AlCl_3$/HCl) 279, 303, 343, 380 (NaOAc) 281, 334,

396 (NaOAc/H_3BO_3)284, 318, 349. ^{1}H-nmr (DMSO) showed at 8.02 (2H, d, H-2',6'), 6.91 (2H, d, H-3', H-5'), 6.79 (1H, s, H-3) and two anomeric protons for two glucose moieties at C-6, C-8, 5.1 (1H, d, H-1") and 4.85 (1H, d, H-1'")

Compound-6: uv λ_{max} (MeOH) 254, 265, 344, (NaOMe) 272, 400, ($AlCl_3$) 271, 300, 345, 410, ($AlCl_3$/HCl) 273, 348, 361, 375 (NaOAc) 268, 404

(NaOAc/H_3BO_3) 258, 360. ^{1}H-nmr (DMSO) showed signal at 7.45 (2 H, d, H-2', H-6'), 6.95 (1 H, d, H-5'), 6.77 (1 H, d, H-8), 6.73 (1 H, s, H-3), 6.4 (1 H, d, H-6) in addition to two anomeric protons for two sugars at 5.1 (1 H, d, H-1" for glucose), 4.55 (1 H, s, H-1'" for rhamnose) and the methyl protons of the rhamnose moiety at 1.07 (3H, d, CH_3 protons).

The insecticidal activity results were shown in table 2 and table 3.

Table 2. Insecticidal activity of different extracts of T. zanonii against the adult of P. oleae (30 beetles/ treatment)

Extracts	Number of dead beetles	% Percentage of mortality
	After 24 h	After 24 h
1- petroleum ether	18 de	60.00
2- fatty alcohols	15 bc	50.00
3- fatty acids M. E.	17 cd	56.67
4- Unsap. Fraction	13 b	43.33
5- Alcoholic	25 f	83.33
6- Aqueous	26 f	86.67
7- Chloroform	20 e	66.67
8- Ethyl acetate	21 e	70.00
9- Butanol	24 f	80.00
10- Untreated control (Without extract)	1 a	3.33

Means marked with the same letters are not significantly different (P<0.05).

DISCUSSION

Cirsiliol: The UV absorption data of compound-1 showed that it is a flavone type with a free OH group at C-4' and no free OH group at C-7. The presence of an *ortho*-dihydroxy system in ring-B was confirmed where there is a hypsochromic shift (54 nm) in band-I in $AlCl_3$/HCl spectrum relative to $AlCl_3$ spectrum. Also it was proved through NaOAc/H_3BO_3 spectrum where there is a bathochromic shift (22 nm) in band-I relative to methanol spectrum.

In the EI-mass spectrum, the presence of the fragment 196 (A^+_1) confirm the presence of the two methoxy groups at ring A.

The ^{13}C-nmr spectrum of compound-1 displayed the most important peaks for 7,6-dimethoxylated flavones in addition to the carbonyl carbon at δ = 182.90 ppm. The two methyl carcons at C-6 and C-7 were appeared at 57.04 and 61.12 repectively,Also these data were coincided with that reported for cirsiliol (18). The other data were shown in table (1). So, compound-1 can be identified as cirsiliol.

Luteolin: All measured data for compound -2 were found to be identical with that reported for luteolin (17).

Chrysoeriol: The UV absorption data of compound-3 proved that it is a flavone type structure with free OH group at C-4' and C-7 without ortho dihydroxy system. The EI-mass spectrum of compound-3 showed a molecular

Table 3. Efficiency of different treatments applied against P. oleae infesting olive trees

Treatment	Mean number/ replicae and % reduction in infestation after sprayir								
	Before treatment	One week		Two weeks		Three weeks		Average	
		M.no	%R	M.no	%R	M.no	%R	M.no	%R
1-Pet. ether extract	14.95	12.00	30.20	13.05	25.59	13.90	24.93	12.98	26.91
2-Fatty alcohols fraction	14.75	12.20	28.07	12.20	29.49	14.00	23.36	12.80	26.97
3-Fatty acids fraction	13.25	11.10	27.16	12.20	21.52	14.00	14.69	12.43	21.12
4-Unsap. fraction	15.00	13.19	19.13	14.05	20.15	15.45	16.83	14.48	18.70
5-Alcoholic extract	14.90	5.85	65.86	6.00	65.67	7.00	62.07	6.28	64.53
6-Aqueous extract	14.75	4.95	70.82	4.95	71.39	5.00	72.63	4.97	71.61
7-Chloroform extract	15.00	6.15	64.35	7.05	59.93	9.40	49.40	7.53	57.89
8-Ethyl acetate extract	14.75	5.95	64.92	7.00	59.54	10.45	42.80	7.80	55.75
9-Butanol extract	13.00	5.00	66.56	5.85	61.64	6.30	60.87	5.72	36.02
10-Control	13.00	14.95	-	15.25	-	16.10	-	15.43	-

Table 1: ^{13}C-nmr data of compound-1,2,6

Carbon No.	δ (ppm)		
	1	2	6
2	165.2	166.39	162.78
3	103.87	103.9	102.98
4	182.9	183.91	181.76
5	153.4	163.26	161.12
6	133.5	100.18	99.42
7	160.64	166.15	164.52
8	92.28	95.05	94.69
9	152.5	159.46	156.81
10	114.26	105.33	105.28
1′	123.58	123.73	121.14
2′	116.84	114.19	113.49
3′	145.6	147.1	145.74
4′	150.21	151.05	150.02
5′	120.48	116.62	116.07
6′	123.58	120.33	119.07
C-6-OCH_3	57.04	-	-
C-7-OCH_3	61.12	-	-
7-O-glucose	-	-	-
1″	-	-	99.83
2″	-	-	73.01
3″	-	-	76.2
4″	-	-	69.5
5″	-	-	75.47
6″	-	-	65.94
7-O-rhamnose	-	-	-
1‴	-	-	100.41
2‴	-	-	70.19
3‴	-	-	70.67
4‴	-	-	71.97
5‴	-	-	68.21
6‴	-	-	17.68

ion peak (M^+) at m/z = 300 (60%) which constituted with the molecular formula $C_{16}H_{12}O_6$. The presence of methoxy group at ring B was confirmed by thr fragment $B1^{\cdot +}$ at m/z = 148.

Finally, the chromatographic and the available spectroscopic data substantiated that compound-3 is chrysoeriol (17).

Xanthomicrol: The UV absorption data confirm the presence of free OH group at C-4' with absence of *ortho*-dihydroxy system and no free OH group at C-7.

The EI-mass spectrum of compound-4 showed a molecular ion peak ($M^{+)}$ at m/z = 344; 6.3% which correspond to the molecular formula $C_{18}H_{16}O_7$. The presence the three methoxy groups at ring-A was confirmed through the presence of the fragment A^+_1 (226) and the fragment at m/z = 118 ; 15.3% .So, we can tentatively identify compound-4 as Xanthomicrol

Apigenin 6, 8-di-O-glucoside: The chromatographic behavour of compound-5 showed, it is aglycosidic in nature. The uv absorption data indicates the presence of a free OH group at C-4' and C-7.

The ^{1}H-nmr revealed the presence of two anomeric protons on ring- A and the other data were coincided with that reported for apigenin (19). After acid hydrolysis, apigenin was isolated as an aglycone and glucose was detected as sugar. The (+ve) FAB/MS of the aglycone of compound-5 displayed a molecular ion peak at m/z = 271 corresponding to the molecular formula of $C_{15}H_{10}O_5$ +1 which coincided with that of apigenin. So, compound-5 can be identified as apigenin 6, 8-di-*O*-glucoside.

Luteolin-7-O-rutinoside: The behaviors of compound-6 in different solvents indicate it is highly glycosidic compound. The UV spectra of compound-6 showed band-I in methanol at 344 nm which proves the flavone nature of this compound.

A bathochromic shift (56 nm) in band-I was noticed upon addition of NaOMe without decreas in intensity indicates the presence of a free OH group at C-4'.

The presence of an *ortho*-dihydroxy system was proved where there is a hypsochromic shift (35 nm) in band-I of $AlCl_3$ spectrum relative to $AlCl_3$/HCl spectrum, also there is a bathochromic shift (16 nm) in band-I of NaOAc/H_3BO_3 spectrum relative to methanol spectrum.

The absence of free OH group at C-7 was confirmed where there is no bathochromic shift in band-II of NaOAc spectrum.

The ^{1}H-nmr data were in accordance with that reported for luteolin 7-*O*-rutinoside.

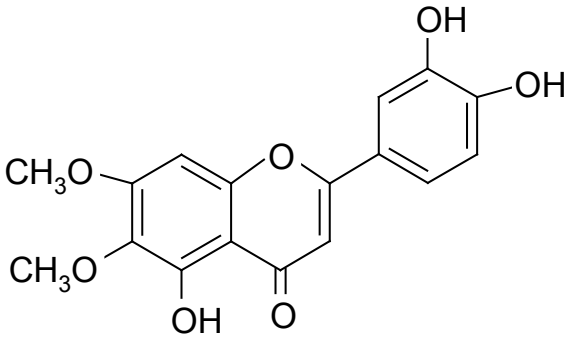
The ^{13}C-nmr spectrum (DMSO) of compound-6 showed the most characteristic signals of flavone diglycoside like C-4 at δ = 181.76, C-1" at 99.83, C-1‴ at 100.41 and C-6‴ of CH_3 group of rhamnose at 17.68. The down filed shift of C-6" (65.94) and C-1‴ (100.41) indicates the two sugars are rutinoside i.e. gluco-(61) rhamnoside (20) and in accordance with those of luteolin-7-*O*-rutinoside. The other data of ^{13}C-nmr were found in table (1).The acid hydrolysis revealed the presence of glucose and rhamnose were detected as sugars and luteolin as an aglycone.

The position of the attachment of these sugars to the aglycone was confirmed at C-7 where the UV spectra of the aglycone showed a bathochromic shift in band-II in NaOAc spectrum relative to methanol spectrum. Also the identity of luteolin was confirmed by the +ve FAB/MS, where it displayed a molecular ion peak at m/z = 287.

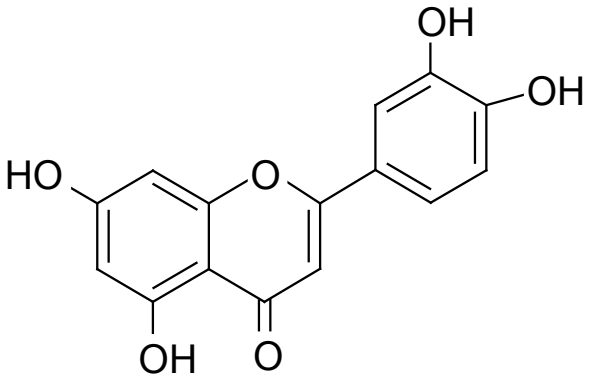
From all the above chromatographic and spectroscopic data, we can identify compound-6 as Luteolin-7-*O*-rutinoside. All these compounds were isolated for the first time from this species.some of it were isolate from other Teucriumspecies like cirsiolol, luteolin, luteolin 7-O-rutinoside, apigenin di glucoside (8, 21–25).

INSECTICIDAL ACTIVITY:

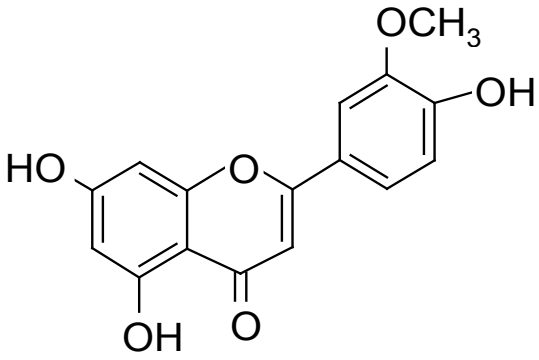
The results in table (2) indicated that, all extracts used proved to have various degrees of insecticidal effect on the adult beetles. There is significance between aqueous



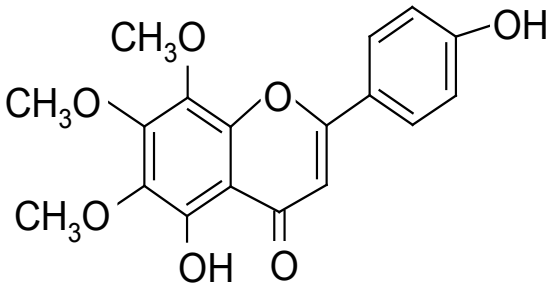
Cirsiliol



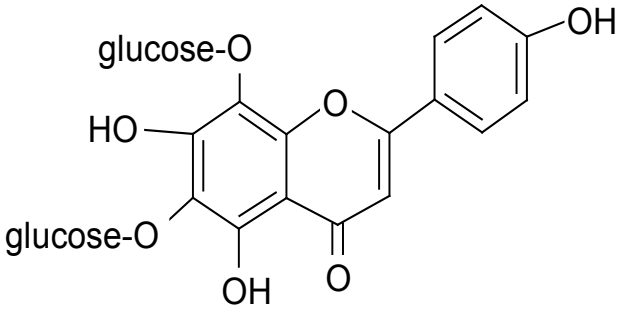
Luteolin



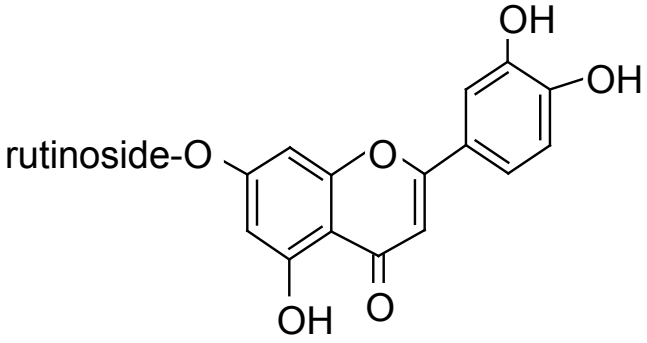
Chrysoeriol



Xanthomicrol



Apigenin 6,8-di-O-glucoside



Luteolin-7-O-rutinoside

and alcoholic extracts. These results clarify that aqueous extract was the most efficient as insecticide followed by alcoholic extract which may be due to the most active compounds like diterpenoids and flavonoids were found in both extracts (26).

Filed experiments results (table 3) of insecticidal activity show that the mean numbers of *P. oleae* Fab. on the olive trees before treatments, ranged from 13:00 to 15:00, indicating a relatively uniform distribution of insect infestation. One week after spraying, the treatments suppressed the levels of infestation to different degrees compared to that of untreated control. Aqueous, alcoholic and butanol extracts significantly lowered the percentage of infestation to 70.82%, 65.86% and 66.56%, respectively. Two weeks post-treatment, aqueous extract become more efficient and had almost similar activity as cidial 50% (conventional chemical insecticides, unpublished data) displaying 71.39% and 73.9% reduction in infestation respectively. Similar results were reported by Ismail and Abdalla (13).

As for the 3rd week after the treatment, both aqueous extract and alcoholic showed good bioresidual activities against *P. oleae* giving 72.63% and 62.07% reduction, respectively. This was in accordance with Masanori *et. al.* in

2000(27), they reported that some methoxylated flavones have antifeedant activity. So the insecticidal activity of T. zanonii may be due to the presence of such compounds in the active extracts. Accordingly, the present study showed that *T. zanonii* extracts was a good candidate to be considered for protecting olive trees against this pest in integrate pest management (IPM) program (28).

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